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Effect of microbial composition on thermophilic acid fermentation

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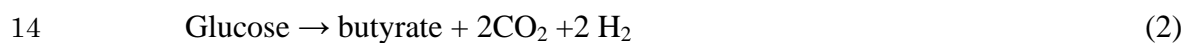
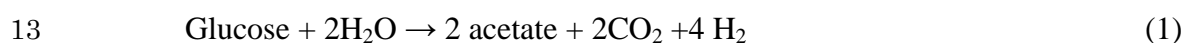
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1. Introduction

Resource recycling and energy-saving systems for processing organic solid waste in urban areas need to be established. Anaerobic digestion has been considered to be a promising energy saving and recovery process for the treatment of organic solid waste with a high water content such as kitchen garbage. Anaerobic digestion is considered to take place in two steps: an acid fermentation phase and a methane fermentation phase. A single-phase system, in which these two steps proceed simultaneously in one reactor, is commonly used because of simplicity in configuration and operation. However, a two-phase system, which consists of separate acid and methane fermentation reactors, has several advantages over conventional single-phase systems [1–3]. Acid fermentation produces many kinds of organic acids such as lactate, butyrate and acetate, and hydrogen gas. Bio-hydrogen production from organic wastes was widely developed and optimal operational conditions such as pH, temperature, substrate and product concentrations, hydraulic retention time (HRT) or solids retention time (SRT) were proposed [4]. Recently, thermophilic condition has been focused on for hydrogen fermentation due to thermodynamic advantage at higher temperature [5]. Akutsu et al. [6] reported that hydrogen production in thermophilic continuous hydrogen fermentation of starch at pH 4.9–5.4 was successful with four different seed sludge, the most predominant microbe was *Thermoanaerobacterium*, but hydrogen production was low and some lactate was produced with one seed sludge due to unknown reason.

1 Shin et al. [7] reported that *Thermoanaerobacterium* and *Desulfotomaculum* were detected in
2 thermophilic hydrogen fermentation at pH 5.5 from food waste but not *Bacillus coagulans*.
3 Ueno et al. [8] reported that *Bacillus* spp. were observed under high OLR conditions in
4 thermophilic hydrogen fermentation using glucose. Ueno et al. [9] reported that no significant
5 lactate was produced at pH 5–8 in thermophilic hydrogen fermentation from organic waste, *B.*
6 *coagulans* was detected at pH 6, and its ratio was lower than *Clostridium* spp. and
7 *Thermoanaerobacterium* spp. While a simple thermophilic L-lactate fermentation of organic
8 wastes under nonsterile conditions using *B. coagulans* has been developed [10–14]. Akao et al.
9 [14] reported that operational conditions of pH 5.5 and 55°C were suitable for L-lactate
10 fermentation of kitchen garbage by indigenous *B. coagulans*, and other bacteria were eliminated
11 under these culture conditions. Typical formulas of hydrogen fermentation from glucose are as
12 follows:



15 Yields of hydrogen by butyrate type fermentation carried out by some *Clostridia* are lower than
16 that of acetate fermentation. Failure in hydrogen fermentation is also widely reported, and one
17 of the typical reasons is lactate production.



1 Hydrogen fermentation is a kind of acid fermentation, and proposed operational parameters for
2 hydrogen fermentation and lactate fermentation are similar. However, key parameters to
3 determine microbial composition and product differences in acid fermentation are still uncertain.

4 Microbial concentrations associated with acid fermentation have been estimated by
5 measurements of Suspended Solids (SS), COD, optical density, protein, and total DNA mass.
6 However, these indices are not appropriate for understanding microbial composition and
7 concentrations in fermentation reactors using complex substrates containing organic particulate
8 materials such as kitchen garbage. Microbial composition can be measured using molecular
9 biology techniques such as random cloning method [15]. Recently, quantification of microbes
10 can be done using quantitative real-time polymerase chain reaction (PCR) with specific primer
11 sets for quantifying specific microbes. The applicability of real-time PCR quantification to
12 evaluate methanogenic behavior has been reported [16, 17]. Li et al. [18] proved the
13 applicability of TaqMan gene probe for real-time monitoring of acidophilic hydrogen-producing
14 bacteria. Wang et al. [19, 20] reported relationship among hydrogen production, growth
15 parameters, and quantitative molecular biological tools. However, they were based on only
16 batch experiments with operational periods of around 120 h. Combined discussion of microbial
17 composition and products is useful in evaluating continuously-operated acid fermentation
18 reactors.

The objective of this study is to investigate relationship between microbial composition and products in an acid fermentation process using kitchen garbage. For this purpose, a continuous thermophilic fermentation experiment was performed using kitchen garbage. HRT and SRT were separately controlled to evaluate their effects on hydrogen and acid fermentation. Microbial composition shift was analyzed using random cloning method, and quantification of *B. coagulans*, which was identified as a key microbe to evaluate the reactor performance, was performed using real-time PCR.

2. Materials and methods

2.1 Reactor and operation

The continuous experiment was performed in a reactor of a 4.5 L working volume in which anaerobic conditions and complete mixing were maintained. Operational conditions are summarized in Table 1. Culture temperature was set at 55°C using a hot water bath and pH was automatically controlled at 6 by the addition of 10 N NaOH (Nisshinrika, NPH-660). Artificial kitchen garbage was prepared by mixing 14 types of foods, based on a survey conducted in Japan [21]. The kitchen garbage comprised 10% cabbage, 10% potato, 10% carrot, 10% radish, 10% Chinese cabbage, 2.5% apple, 7.5% orange peel, 10% banana peel, 10% boiled rice, 2.5% bread, 7.5% noodles, 2.5% boiled eggs, 2.5% meat, and 5% fish. The characteristics of this raw

garbage are summarized in Table 2. Materials were mixed, milled into a slurry and stored in a freezer until use. This garbage was diluted two-fold and used for the acid fermentation experiment. The operation was continued in a fill-and-draw style once a day, and samples were taken just before feeding. Seed sludge was originally obtained from a thermophilic digester plant of kitchen garbage, and then it was cultured for about 5 years using similar kitchen garbage in our laboratory.

2.2 Chemical analysis

Lactate and other organic acids were analyzed using High Performance Liquid Chromatography (HPLC) (Organic acids analysis system, column; Shima-pack SCR-102H, Shimadzu, Kyoto, Japan). Carbohydrate concentration, expressed using glucose concentration as standard, was determined using a phenol–sulfate method [22]. Protein concentration was determined using Lowry method [23]. Carbon dioxide, hydrogen and methane were analyzed using CG-14B (Shimadzu). Total solids (TS), SS, volatile solids (VS), total and soluble COD (TCOD and SCOD, respectively) were analyzed according to the Standard Method [24]. Total nitrogen was analyzed using Auto Analyzer II (BRAN+LUEBBE, Norderstedt, Germany) after digestion by peroxydisulfuric acid. Dissolved samples were prepared by filtration through a cellulose acetate filter (pore size = 0.45 μm).

2.3 Molecular biological analysis

1 The microbial community was analyzed using random cloning method [15]. 16S rRNA gene
2 was extracted using a DNeasy Tissue Kit (Qiagen, Hilden, Germany). A primer set of
3 UNIV519F (5'-cagcmgccgcggtaatwc-3') and UNIV1406R (5'-acgggcggtgtgtrc-3') [25] for most
4 bacteria and archaea was used to amplify approximately 900 and 700-bp fragments, respectively.
5 Polymerase chain reaction (PCR) conditions (PTC-200 DNA Engine, MJ research, Watertown,
6 MA, USA) were at 94°C for 5 min, followed by 30 cycles of at 94°C for 1 min, at 68°C for 1 min
7 and at 72°C for 1 min. The final extension step was at 72°C for 5 min. The amplified 16S rRNA
8 gene was separated by 2% (w/v) agarose gel (SeaKem GTG Agarose, Cambrex, Rockland, ME,
9 USA) electrophoresis run at 135 V for 45 min. 16S rRNA gene products were purified with
10 QIAquick Gel Extraction Kit (Qiagen). The purified products were ligated into pCR4-TOPO
11 vector (Invitrogen, Carlsbad, CA, USA) and then transformed into *Escherichia coli* TOP10
12 competent cells. Colonies were screened on LB-based agar plates with Kanamycin, X-gal, and
13 IPTG. Cells from randomly selected white colonies were assayed on an LB medium at 37°C for 1
14 hour. The plasmids were extracted using QIAprep Spin Miniprep kit (Qiagen) and entreated
15 sequencing of 16S rRNA gene at the TaKaRa Bio Dragon Genomics Center (Yokkaichi, Japan).
16 Obtained sequence data were compared with similar sequences in National Center for
17 Biotechnology Information data using the BLAST program [26]. All clones having a sequence
18 similarity of more than 97% with each other were grouped into an operational taxonomic unit

1 (OTU).

2 For real-time PCR reactions, LightCycler 1.2 (Roche, Mannheim, Germany) was used to
3 quantify the target microbes [27]. A primer pair of BACO186F (5'-gcatggaggaaaaaggaa-3') and
4 BACO447R (5'-cccggcaacagagtttta-3') for quantification of *B. coagulans* was developed
5 (Asahira T. Quantification of identified microbes in thermophilic methane fermentation reactors
6 based on 16S rDNA. Master thesis, Kyoto University 2006). The primer set of UNIV519F and
7 UNIV1406R [25] was used for total bacterial measurement. All reactions used 20 μ L reaction
8 capillary tubes with the LightCycler FastStart DNA MasterPlus SYBR Green I (Roche). Each
9 capillary tube was separately loaded with 1 μ L of sample 16S rRNA gene, followed by addition
10 of 1 μ L (final concentration of 0.5 μ M) of forward and reverse primers along with 2 μ L of SYBR
11 Green I Master mix, and PCR-grade sterile water to a final volume of 10 μ L. A negative control
12 without the corresponding template 16S rRNA gene was included in each real-time PCR reaction
13 for each primer pair. Amplification of the target 16S rRNA gene used the following conditions:
14 initial 10 min incubation at 95°C for Taq polymerase activation; 30 cycles of denaturation at
15 95°C for 10 s, annealing at 56°C (*B. coagulans*) or 68°C (total bacterial) for 10 s, and extension
16 at 72°C for 30 s. The transition rate was 20°C/s for all segments during cycling. Cycle threshold
17 (*C_t*) was determined by fit point method using LightCycler software (version 3.5, Roche).
18 Standard 16S rRNA gene concentrations were measured using a spectrophotometer (NanoDrop

1 ND-1000, Wilmington, DE, USA). Each 16S rRNA gene concentration was determined from the
2 *Ct* obtained from regression equations of the external standards.

3

4 **3. Results**

5 *3.1 Fermentation performances*

6 Time courses for the continuous operation are shown in Figure 1. Other SCOD indicates SCOD
7 excluding organic acids, and it was calculated by the difference between SCOD and sum of
8 organic acids measured. TCOD and SCOD were around 140 and 90 gCOD/L, respectively.
9 Particle material and total organic acid concentrations were almost constant during this
10 experiment. This means that solubilization efficiency of particle COD was stable. Other SCOD
11 increased to around 10 gCOD/L in Run 3, which showed that solubilization was not affected but
12 acidification was delayed and SCOD with larger molecular size remained under low SRT
13 condition. Biogas produced from acid fermenter was mainly composed of CO₂ and H₂, and
14 methane was not detected during the operation. Acetate, propionate and valerate were almost
15 less than 5 gCOD/L, and butyrate and lactate were the predominant products. Skiadas et al. [28]
16 proposed a model which shows that glucose is converted into lactate or unknown intermediate
17 product, and then they are converted into propionate and acetate, as lactate is a key intermediate
18 of glucose fermentation, especially under high loading condition. In the present study, the HRT

was set at 3.5 d, and high loading condition was maintained. Then, acidification did not reach to propionate and acetate, which would be the substrate of methanogenesis. At the beginning of Run 2, product composition changed a lot, but products at the end of Run 2 and at the beginning of Run 3 were similar. This meant that SRT 10.5 d in Run 1 might be the critical value to decide the product difference from SRT 7 d in Run 2 and SRT 3.5 d in Run 3. Figure 2 shows the average organic acids and hydrogen production in Run 1–3. Organic acids composition, especially butyrate and lactate, changed greatly during the experiment. Butyrate fermentation occurred under the longer SRT condition in Run 1. When SRT was set at shorter in Run 3, lactate production increased, and butyrate and hydrogen production decreased. Noike et al. [29] reported that lactate producing bacteria and hydrogen producing bacteria were competitive for substrate and their products showed reverse relationship. This corresponds with the present study in that lactate production increased and hydrogen production decreased simultaneously. SRT was the important parameters to control organic acids production and bio-hydrogen production in acid fermentation.

3.2 Microbial composition

A summary of each microbial composition analysis is shown in Table 3. Most OTUs were *Firmicutes*, and just a few OTUs were *Actinobacteria*, *Bacteroidetes*, *Proteobacteria* and *Thermotogae*. In Run 1, 78% OTUs were closely related to *Clostridium* spp. with average

1 similarity of 97%. In Run 2, about half OTUs were closely related to *Bacillus* spp., but not
2 related to *B. coagulans*, and the other half OTUs were closely related to *Clostridium* spp. Then
3 in Run 3, most OTUs were closely related to *B. coagulans* with average similarity of 99%.
4 OTUs closely related to *Lactobacillus* spp. with average similarity of 99% were also detected in
5 *Firmicutes*, but their occupying ratio was low. From Run 1 through Run 3, microbial
6 composition change from *Clostridium* spp. to *Bacillus* spp., especially *B. coagulans* was
7 observed. In our reactor, *Thermoanaerobacterium* was detected only in one sample in Run 3,
8 and was not the key microbe for hydrogen production unlike other researches using food wastes.

9 Time course results for lactate and butyrate concentrations, and *B. coagulans* and
10 *Clostridium* spp. ratio measured using random cloning are shown in Figure 3. Product shift from
11 butyrate to lactate and microbial composition shift from *Clostridium* spp. to *B. coagulans* were
12 simultaneously observed. The relationship between *B. coagulans* ratio measured using random
13 cloning and organic acid concentrations is shown in Figure 4. Clear relationship between *B.*
14 *coagulans* ratio and lactate concentration was obtained with $R^2=0.7$. Akao et al. [13] reported
15 results of two microbial composition analysis using random cloning in the semi-continuous
16 fermentation of kitchen garbage at pH 6 and 55°C under SRT = HRT operation. *B. coagulans*
17 ratio was both approximately 80% and lactate concentration was both approximately 40 gCOD/L.
18 These values also fit this relationship. When lactate concentration increased, butyrate

concentration decreased, and there was no obvious change in the other organic acid concentrations such as acetate and propionate. The relationship between *Clostridium* spp. ratio measured using random cloning and organic acid concentrations is shown in Figure 5. Irreversible relationship between *Clostridium* spp. and lactate accumulation was found having $R^2=0.7$, which was opposite to that between *B. coagulans* and lactate.

3.3 Applicability of real-time PCR quantification for *B. coagulans*

Time courses for 16S rRNA gene concentrations measured using real-time PCR are shown in Figure 6. Total 16S rRNA gene concentration was stably maintained at around 10^{12} copies/L, and 16S rRNA gene concentration of *B. coagulans* varied in the range of 10^8 – 10^{11} copies/L. Hidaka et al. [27] reported that the average ratio of 16S rRNA gene concentration to mass of *B. coagulans* was 6.47×10^{10} copies/gCOD-biomass in the batch experiment of *B. coagulans* using substrate mainly containing glucose. Using this ratio, in Run 3, *B. coagulans* concentration was estimated to be approximately 3.2 gCOD-biomass /L. It is equivalent to 2.3 gVS-biomass /L, given that bacterial chemical formula is $C_5H_7O_2N$.

The relationship between *B. coagulans* ratio measured using random cloning and real-time PCR is shown in Figure 7. Although the ratio measured using random cloning was nine times higher than that measured using real-time PCR, clear relationship was obtained with $R^2=0.9$. 16S rRNA gene extraction from the samples was same, but after that each procedure

was different, and detection efficiency might be different. This clear relationship proved that both methods can detect change in *B. coagulans* dominance. The relationship between *Bacillus* spp. ratio measured using random cloning and *B. coagulans* ratio measured using real-time PCR is also shown in Figure 7. Unclear relationship with $R^2=0.5$ was observed compared to the previous one. This also verified the specificity of the developed primer for quantification of *B. coagulans*. The relationship between 16S rRNA gene concentrations for *B. coagulans* measured using real-time PCR and lactate concentrations is shown in Figure 8. Clear relationship between 16S rRNA gene concentrations for *B. coagulans* and lactate concentration was also obtained with $R^2=0.4$. The results of real-time PCR varied logarithmically, and R^2 value could be low, but relationship was obvious. Therefore, real-time PCR can be used to monitor change in *B. coagulans* in kitchen garbage fermentation, where complex organic components are included.

4. Discussion

There are many literatures on thermophilic hydrogen fermentation from carbohydrate. Typical proposed optimum conditions for thermophilic hydrogen fermentation from kitchen garbage or other carbohydrate-rich organic wastes are reported to be around pH 5–6 [6, 7, 9, 30–33]. Failure of hydrogen fermentation by lactate production was explained by high loading rate [8, 30, 34, 35]. During our continuous experiment, the substrate, HRT, OLR and particle COD

concentrations were almost constant, but SRT was different. In Run 1, where hydrogen production was the highest, SRT was three times higher than HRT. This was possibly helpful to maintain *Clostridium* spp. and hydrogen production.

Akao et al. [13] proposed the condition of pH 5–6 and 55°C for semi-continuous lactate fermentation by *B. coagulans*. *B. coagulans* is included in kitchen garbage, and seeding sludge is not required [14]. Lee et al. [36] reported that lactate and no hydrogen were produced at pH 5.5 and hydrogen was produced at pH 6 or over in fermentation of vegetable kitchen wastes under a thermophilic condition with seed sludge from kitchen-waste compost. In practical thermophilic hydrogen fermentation, there must be a risk of failure in hydrogen production by indigenous *B. coagulans*. Noike et al. [29] reported the consumption of reducing powers to be used for hydrogen gas generation by lactate bacteria of *Lactobacillus* on mesophilic hydrogen fermentation by *Clostridium*, and suggested heat treatment for reduction of hydrogen production inhibition. Some other pre-treatment methods including heat, acid and base were proposed for mesophilic hydrogen fermentation of food waste without inoculum addition to kill H₂-consuming microorganisms [37]. However, both most *Clostridium* spp. and *B. coagulans* are spore-forming, and pre-treatment could not be effective to prevent proliferation of *B. coagulans* in thermophilic hydrogen production. Antibiotics produced during lactate fermentation might inhibit hydrogen fermentation [38]. *B. coagulans* produces antibiotics [39, 40], and this characteristic possibly

1 made the dominance of *B. coagulans*. The growth rate of *B. coagulans* considering the
2 inhibition by a lactate concentration of 25 gCOD/L, like in Run 1, is estimated to be 0.26 (1/h)
3 [27]. Collet et al. [41] reported continuous thermophilic hydrogen production by *Clostridium*
4 *thermolacticum* under dilution rate of 0.0013–0.19 (1/h), and it could be maintained under higher
5 dilution rate. O-Thong et al. [42] reported that *Thermoanaerobacterium thermosaccharolyticum*
6 PSU-2 in *Clostridia* had maximum specific growth rate of 0.31 (1/h) in thermophilic hydrogen
7 production. Therefore, HRT 3.5 d was sufficient to retain *Clostridium* spp. and
8 *Thermoanaerobacterium* spp. in the reactor, if there were no adverse effects by such as pH, and
9 substrate and product concentrations. Low dominance of *Clostridium* spp. in Run 3 and low
10 dominance of *Thermoanaerobacterium* spp. in Runs 1–3 could be due to inhibition of antibiotics
11 produced by *B. coagulans*. Chu et al. [43] recirculated mesophilic methane fermenting sludge
12 for continuous thermophilic hydrogen fermentation at pH 5.5 from food waste. Lee et al. [44]
13 recirculated thermophilic methane fermenting sludge for continuous thermophilic hydrogen
14 fermentation at pH 5.0–5.7 from food waste. Luo et al. [33] showed that pre-treatment of
15 anaerobic sludge is unnecessary for practical thermophilic hydrogen fermentation from cassava
16 stillage with seed sludge from a full-scale mesophilic upflow anaerobic sludge blanket reactor.
17 In these researches, the substrates could include *B. coagulans*, but continuous hydrogen
18 production was maintained successfully. Returning or seeding sludge which include bacteria

other than *B. coagulans* might be the key operation to prevent growth of *B. coagulans*. In Run 1, high SRT was helpful to retain sufficient *Clostridium* spp. compared to *B. coagulans* included in the substrate. Operation of higher SRT than HRT could lead to such an effect as sludge return, and this possibly resulted in maintaining sufficient *Clostridium* spp. Operational conditions such as OLR, pH, temperature, HRT, SRT and sludge return are essential parameters to decide microbial composition, which affects fermentation products.

Importance of microbial composition monitoring in hydrogen and acid fermentation was presented, and applicability of real-time PCR quantification was proved. Real-time PCR can be applied easier in terms of cost and time than random cloning. Wang et al. [20] reported that 16S rRNA genes did not exhibit any positive relationship with hydrogen production, and hydrogen production was lineally proportional to the level of functional gene (*hydA*) expression in the batch experiments. However, our semi-continuous experiment proved the applicability of 16S rRNA gene quantification for monitoring bacterial community changes in dark fermentation. In batch cultivation, growth dynamics could be different in each growth phase, such as lag phase and logarithmic phase [45]. In stably operated continuous operation, this effect is minimized, and real-time PCR quantification of 16S rRNA can be applied to evaluate microbial composition. Real-time PCR quantification methods would be useful for developing a new monitoring tool in dark fermentative biohydrogen, especially with complex substrates, where many kinds of

1 bacteria compete with each other in the same reactor.

2

3 **5. Conclusions**

4 In our continuous fermentation using kitchen garbage, butyrate and hydrogen fermentation
5 occurred under the longer SRT condition, and when SRT was set at shorter, lactate production
6 increased, and butyrate and hydrogen production decreased. Simultaneously, microbial
7 composition shift from *Clostridium* spp. to *Bacillus* spp., especially *B. coagulans* was observed
8 and clear relationship between *B. coagulans* ratio and lactate concentration was obtained.

9 Returning or seeding sludge which include bacteria other than *B. coagulans* might be the key
10 operation to prevent growth of *B. coagulans*. Applicability of real-time PCR quantification to
11 monitor change in *B. coagulans* in kitchen garbage fermentation was verified. Real-time PCR
12 quantification methods would be useful for developing a new monitoring tool for acid
13 fermentation, especially with complex substrates, where many kinds of bacteria compete with
14 each other in the same reactor.

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- 7

1 Table 1 Experimental condition

		Run 1	Run 2	Run 3
Period	(d)	0–25	26–110	111–300
HRT	(d)	3.5	3.5	3.5
SRT	(d)	10.5	7	3.5
OLR	(gCOD _{Cr} /(L-reactor·d))	43	43	43
pH	(–)	6	6	6

2

3

1 Table 2 Characteristics of raw kitchen garbage

	Total	Soluble
TS (g/L)	189	—
SS (g/L)	120	—
VS (g/L)	179	—
COD (g/L)	292	104
Carbohydrate (g/L as glucose)	145	79
Protein (g/L as albumin)	55	15
T-N (mgN/L)	4.8	1.7
pH (-)	4.8	—

2

1 Table 3 Summary of microbial diversity analysis

Phylum	Order	Genus	Average similarity	day									
				Run 1	Run 2				Run 3				
				25	45	67	82	103	124	153	180	219	
Number of clone analyzed			91	94	93	93	92	104	95	93	94		
Actinobacteria								1			1		
Bacteroidetes											1		
Firmicutes	Bacillales	Bacillus coagulans	99%	3			1		93	89	88	69	
		Bacillus spp.	99%	10	50		34	82		1	3	9	
		[others]					3					1	
	Clostridia	Clostridium spp.	97%	78	37	91	42	12				1	19
		Thermoanaerobacterium spp.	100%									1	
		[others]		1	4	2	13	2					
	Lactobacillales	Lactobacillus spp.	99%	4	4	4			1	7			1
		[others]				1	2				2	2	1
Proteobacteria					1		2	3	1		2		
Thermotogae									4				
Uncertain				3	3	1	2		1				

- 2
- 3 detected ratios expressed as (%)

Figure 1 Time course results for the continuous operation. (a) TCOD, SCOD, total organic acid, butyrate and lactate; (b) acetate, propionate, valerate and other organic acids.

Figure 2 Average organic acid and hydrogen production (average \pm SD).

Figure 3 Time course results for lactate and butyrate concentrations, and *B. coagulans* and *Clostridium* spp. ratio measured using random cloning.

Figure 4 Relationship between *B. coagulans* ratio measured using random cloning and organic acid concentrations.

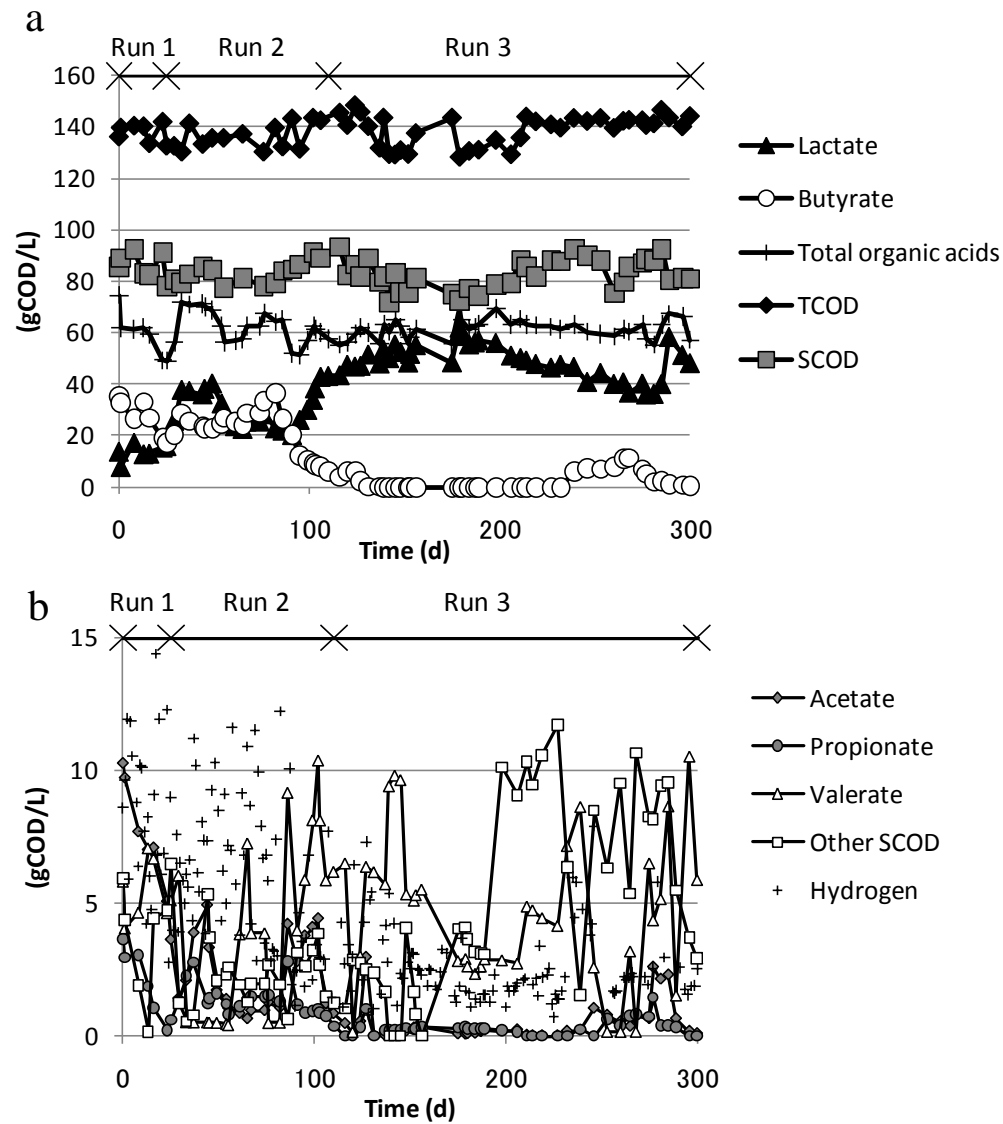
Figure 5 Relationships between *Clostridium* spp. ratio measured using random cloning and organic acid concentrations.

Figure 6 Time course results for 16S rRNA gene concentrations.

Figure 7 Relationship between *B. coagulans* or *Bacillus* spp. ratio measured using random cloning and *B. coagulans* ratio measured using real-time PCR.

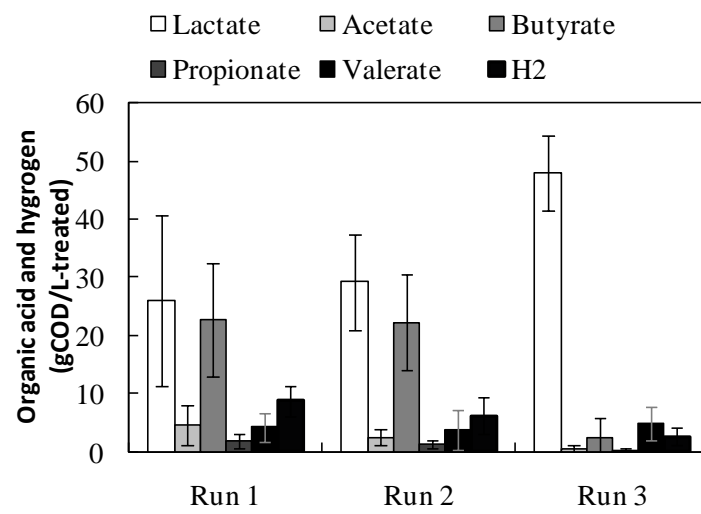
Figure 8 Relationship between 16S rRNA gene concentrations for *B. coagulans* measured using real-time PCR and lactate concentrations.

1 Figure 1



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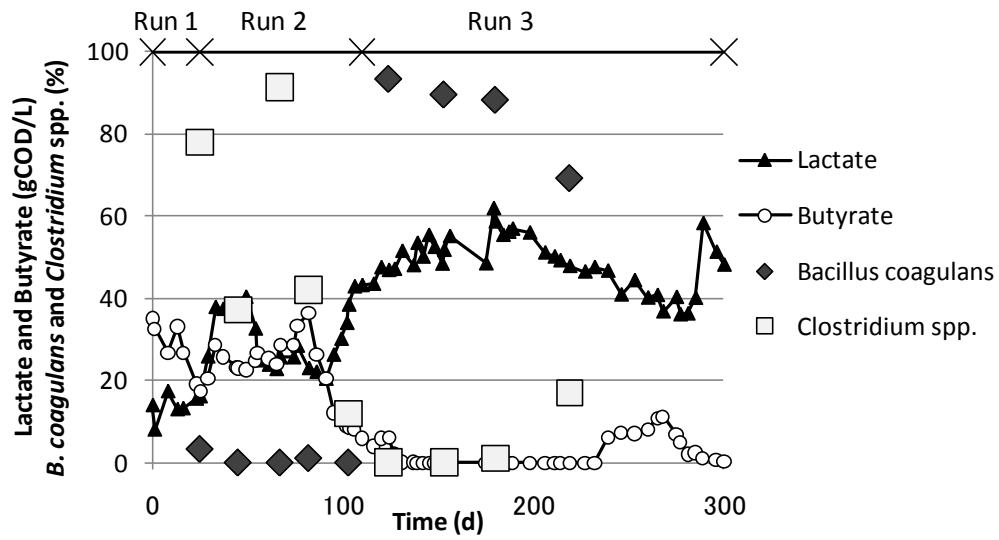
1 Figure 2



2

3

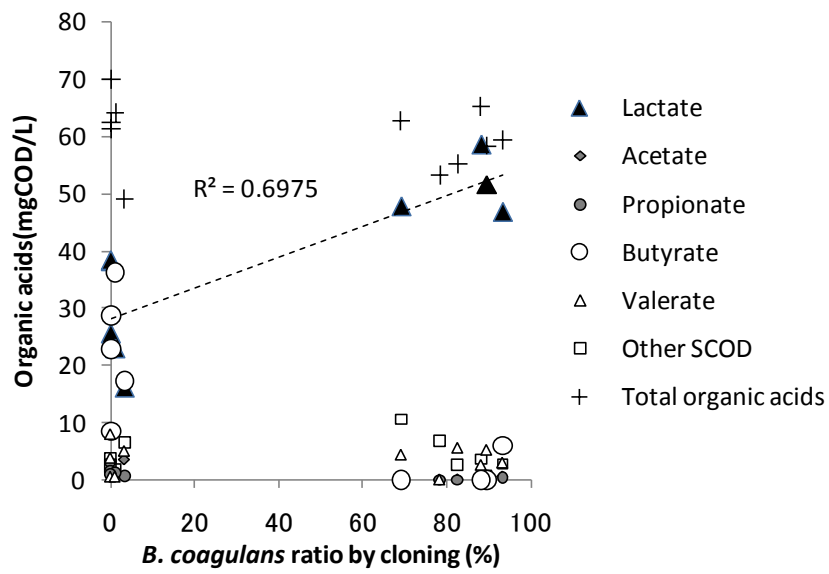
1 Figure 3



2

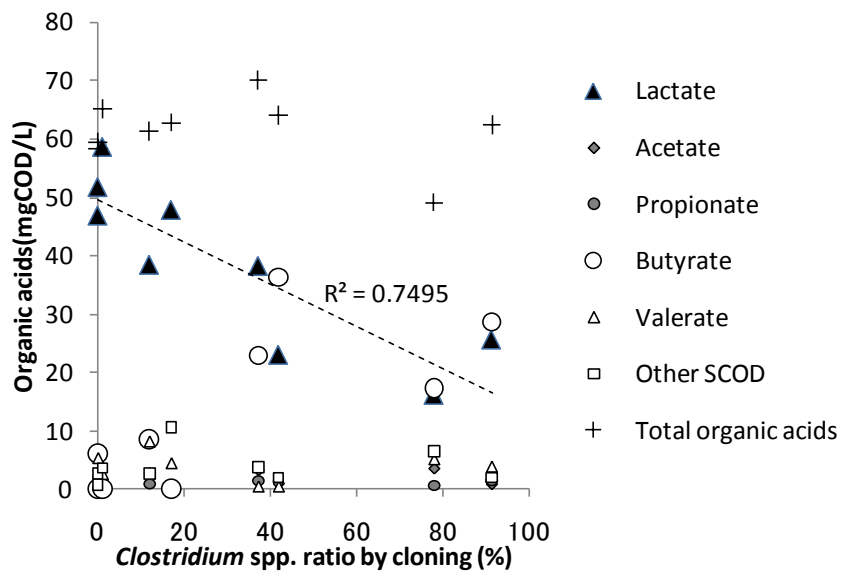
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1 Figure 4



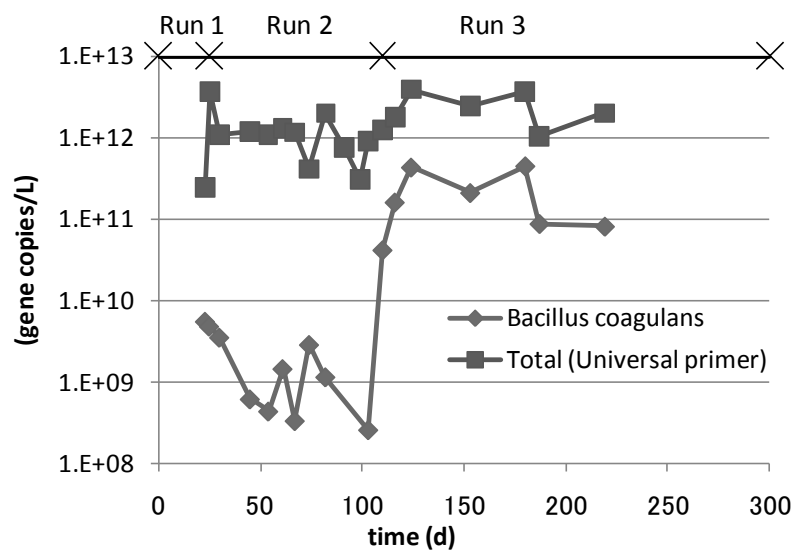
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1 Figure 5



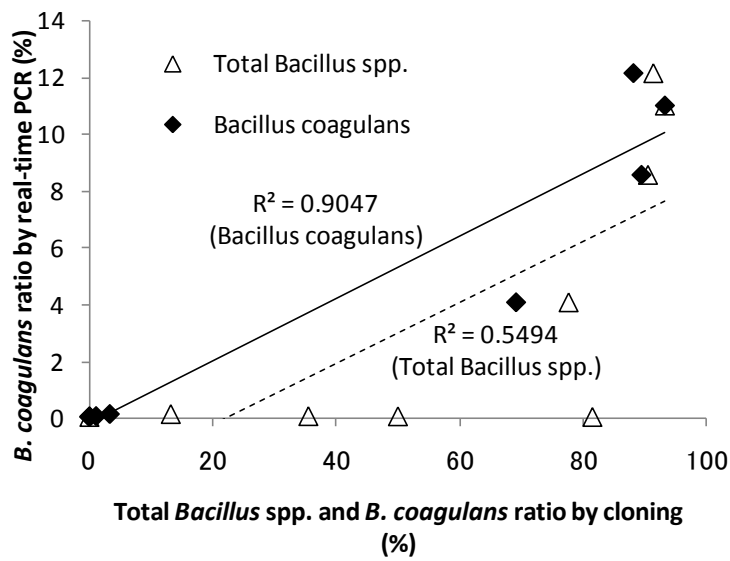
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1 Figure 6



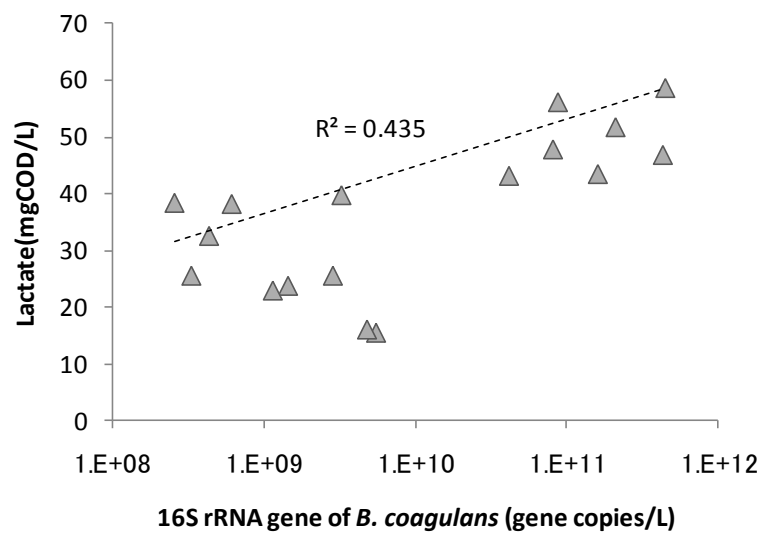
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1 Figure 7



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1 Figure 8



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